FORM PTO-1390 (REV 10-95)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371		MERCK 2289
		U.S. APPLICATION NO. (If known, see 37 CFR §1.5) **09/913494**

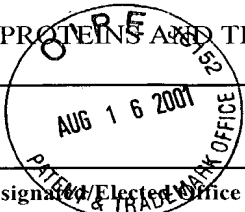| INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/00978	8 FEBRUARY 2000	19 FEBRUARY 1999

TITLE OF INVENTION

GLUCOSE DEHYDROGENASE FUSION PROTEINS AND THEIR UTILIZATION IN EXPRESSION SYSTEMS
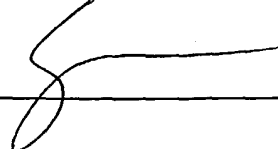
APPLICANT(S) FOR DO/EO/US

LINXWEILER, Winfried, et al .

[stamp: AUG 1 6 2001 PATENT & TRADEMARK OFFICE]

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ■ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.

3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).

4. ■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ■ A copy of the International Application as filed (35 U.S.C. §371(c)(2))

    a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).

    b. ■ has been transmitted by the International Bureau.

    c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ■ A translation of the International Application into English (35 U.S.C. §371(c)(2)).

7. ■ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))

    a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).

    b. ☐ have been transmitted by the International Bureau.

    c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.

    d. ■ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).

9. ■ An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included.

13. ■ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

(November 1998)

U.S. APPLICATION NO. (if known, see 37 CFR §1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/913494	PCT/EP00/00978	MERCK 2289

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)):

Search Report has been prepared by the EPO or JPO...	$860.00
International preliminary examination fee paid to USPTO (37 CFR §1.482).........	$690.00
No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2)).......................	$710.00
Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO............................	$1000.00
International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)................................	$100.00

	CALCULATIONS	PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. §1.492(e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	0	x $ 18.00	$0.00	
Independent claims	1 - 3 =	0	x $ 80.00	$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 270.00		
TOTAL OF ABOVE CALCULATIONS =				$860.00	

Reduction of 1/2 for filing by small entity, if applicable. A Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§1.9, 1.27, 1.28).

SUBTOTAL =	$860.00	

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. §1.492(f)).

TOTAL NATIONAL FEE =	$860.00	

Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). $40.00 per property.

TOTAL FEES ENCLOSED =	$860.00	
Amount to be refunded:		
charged:		

a. ■ A check in the amount of $860.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 13-3402 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ■ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-3402. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO: Customer Number 23,599

23599

PATENT TRADEMARK OFFICE

Filed: 16 August 2001

AJZ:kmo

SIGNATURE

Anthony J. Zelano
NAME

27,969
REGISTRATION NUMBER

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

International Application No.   :   PCT/EP00/00978

International Filing Date   :   8 FEBRUARY 2000

Priority Date(s) Claimed   :   19 FEBRUARY 1999

Applicant(s) (DO/EO/US)   :   LINXWEILER, Winfried, et al.

Title: GLUCOSE DEHYDROGENASE FUSION PROTEINS AND THEIR
        UTILIZATION IN EXPRESSION SYSTEMS

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

SIR:

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

IN THE CLAIMS:

4.   (Amended) DNA, characterized in that it codes for a fusion protein according to Claim 1.

7.   (Amended) Use of glucose dehydrogenase as detector protein for any recombinant protein/polypeptide X in a fusion protein according to Claim 1.

8.   (Amended) Use of glucose dehydrogenase in a detection system for the expression of a recombinant protein/polypeptide X as constituent of a fusion protein according to Claim 1.

9.   (Amended) Use of glucose dehydrogenase for detecting protein-protein interactions, where one partner corresponds to the recombinant protein/polypeptide X in Claim 1.

10.   (Amended) Use of glucose dedydrogenase in a fusion protein according to Claim 1 as detector protein for any third protein/polypeptide which is not a constituent of the

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fusion protein according to Claim 1 and is able to bind to the second sequence of the protein/polypeptide X in the said fusion protein.

13.   (Amended) Method for the rapid detection of any recombinant protein/polypeptide X by gellectrophoresis, characterized in that a fusion protein according to Claim 1 is prepared and fractionated by gel electrophoresis, and the recombinant protein/polypeptide to be detected in the gel is visualized via the enzymic activity of glucose dehydrogenase.

17.   (Amended) Method according to Claim 13, characterized in that the specific staining of the glucose dehydrogenese is followed by a general protein staining.

REMARKS

The purpose of this Preliminary Amendment is to eliminate multiple dependent claims in order to avoid the additional fee. Applicants reserve the right to reintroduce claims to canceled combined subject matter.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached pages are captioned "**Version With Markings to Show Changes Made**".

Respectfully submitted,

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AJZ:jmm

FILED: 16 AUGUST 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claims 4, 7-10, 13 and 17 have been amended as follows:

4.   (Amended) DNA, characterized in that it codes for a fusion protein according to Claims 1~~-3~~.

7.   (Amended) Use of glucose dehydrogenase as detector protein for any recombinant protein/polypeptide X in a fusion protein according to Claims 1 ~~to 3~~.

8.   (Amended) Use of glucose dehydrogenase in a detection system for the expression of a recombinant protein/polypeptide X as constituent of a fusion protein according to Claims 1 ~~to 3~~.

9.   (Amended) Use of glucose dehydrogenase for detecting protein-protein interactions, where one partner corresponds to the recombinant protein/polypeptide X in Claims 1 ~~to 3~~.

10.   (Amended) Use of glucose dedydrogenase in a fusion protein according to Claims 1~~-3~~ as detector protein for any third protein/polypeptide which is not a constituent of the fusion protein according to Claims 1~~-3~~ and is able to bind to the second sequence of the protein/polypeptide X in the said fusion protein.

13.   (Amended) Method for the rapid detection of any recombinant protein/polypeptide X by gellectrophoresis, characterized in that a fusion protein according to Claims 1 ~~to 4~~ is prepared and fractionated by gel electrophoresis, and the recombinant protein/polypeptide to be detected in the gel is visualized via the enzymic activity of glucose dehydrogenase.

17.   (Amended) Method according to Claims 13 ~~to 16~~, characterized in that the specific staining of the glucose dehydrogenese is followed by a general protein staining.

Glucose dehydrogenase fusion proteins and their use in expression systems

The invention relates to novel recombinant fusion
5   proteins which comprise as one constituent a protein
sequence having the biological activity of glucose
dehydrogenase (GlcDH), and to their use for the simple
and efficient detection of any proteins/polypeptides,
which preferably serve as fusion partners, and for the
10   rapid optimization of expression systems which are able
to express the said proteins/polypeptides.

In this regard, GlcDH or the sequence having the
biological activity of GlcDH assumes the role of a
15   marker or detector protein. A particular characteristic
of this enzyme is exceptional stability to denaturing
agents such as SDS. GlcDH as marker or detector protein
shows undiminished enzymatic activity even after the
reducing and denaturing conditions of SDS-PAGE gels.
20   Fusion proteins comprising GlcDH can therefore be
detected using a sensitive enzymatic reaction based on
this surprising behaviour. It is thus also possible
with GlcDH as marker for the required expressed protein
to be detected rapidly, at low cost and efficiently.
25

It is furthermore possible in a number of cases for
(GlcDH-protein/polypeptide fusion proteins to be
expressed in higher yield and stability, especially in
E. coli, than without GlcDH. Corresponding fusion
30   proteins can thus be used per se for obtaining and
preparing proteins/polypeptides.

The *in vivo* expression of recombinant proteins is
playing an ever increasing part in biotechnology. The
35   ability to obtain, purify and detect cloned gene
products from pro- and eukaryotic expression systems
such as, for example, bacterial, yeast, insect or
mammalian cells is frequently also used for studies of

protein structure and function, of protein-protein and protein-DNA interactions, and antibody production and mutagenesis. It is possible with the aid of the DNA recombination technique to modify natural proteins

5 specifically to improve or alter their function. The recombinant proteins are synthesized in expression systems which are continually being further developed and which can be optimized at many different points in the system.

10

The overall process of recombinant protein synthesis can be divided into two sections. In a first step there is molecular biological isolation of the gene and expression of the target protein, and in the next step

15 there is detection and purification from the recombinant cells or their growth medium. At the molecular level, the gene of a protein is cloned into an expression vector provided for this purpose and then inserted into a host cell (pro- or eukaryotic cell) and

20 expressed therein. Bacterial cells prove in this connection to be simple and cost-effective systems affording high yields. The host cell most frequently employed is the Gram-negative bacterium E. coli.

25 The aim of expression of foreign genes in *E. coli* is to obtain the largest possible amount of bioactive recombinant proteins, which is called overexpression. It is known that eukaryotic foreign proteins may lose their biological activity during this through

30 aggregation, as inclusion bodies, through incorrect folding or proteolytic degradation. One possibility of avoiding these frequently occurring difficulties is for the expressed proteins to be expelled from the cell as secreted proteins or else for so-called fusion proteins

35 to be used, through which insoluble recombinant proteins may be present in soluble form in the cell.

In order to investigate the function of proteins and their interaction partners which are important for the

function, proteins are usually expressed in eukaryotic cells. The post-transcriptional modifications which are important for the function, and the correct compartmentation can take place therein. In addition, other proteins important for the correct folding and processing are present.

Eukaryotic expression systems are also appropriate for expressing relatively large proteins and proteins which require post-transcriptional modifications such as, for example, S-S bridge formation, glycosylation, phosphorylation etc. for correct folding. Since these systems are usually complicated and costly, and the expression rate is below that of E. coli, it is particularly important to have a detection system which is rapid, reliable, sensitive and reasonably priced.

Numerous gene fusion systems exist for detecting foreign proteins which have been formed by recombination and whose biological function is unknown. In these, the expressed fusion protein is detected via the fusion protein portion whose function is known.

A sensitive detection system is necessary in order to determine correct expression, the amount expressed, the molecular weight and the functional activity of the fusion protein formed. The number of proteins of unknown function is increasing rapidly and it is becoming increasingly important to develop rapid and cost-effective detection systems therefor. With most gene fusion systems, immunological methods such as, for example, the enzym-linked immunosorbent assay (ELISA) or the Western blot are employed, in which fusion proteins formed by recombination are detected with the aid of specific antibodies.

However, corresponding fusion proteins not only have the described advantage that the foreign protein can easily be detected and analysed indirectly; on the

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contrary in many cases they allow the required protein to be expressed in higher yields than would be the case without its fusion partner. Each fusion partner has advantages, which it is not uncommonly able to transfer
5 to the other partner, in a particular expression system. Thus, for example, the sensitivity of some proteins to protolytic [sic] degradation can be reduced when it is [sic] in the form of a fusion protein. Fusion proteins also frequently have more favourable
10 solubility and secretion properties than the individual components.

There are thus numerous reasons for carrying out gene fusions for expressing recombinant proteins in
15 heterologous hosts. These are: increasing the solubility of foreign proteins, increasing the stability of soluble foreign proteins, localizing the foreign protein in a specific section of the cell, rapid isolation of foreign proteins by simplified
20 purification strategies, possibility of the fusion protein to be specifically cleaved off, possibility of rapid detection of the foreign protein from unpurifed cell extracts.

25 At present there are many functional tests for testing the expression of recombinant proteins with the aid of gene fusion systems. These comprise simple tests which usually make direct detection possible from unpurified cell extracts. However, the test systems differ
30 considerably in the time taken, throughput and sensitivity.

For the abovementioned purposes it is possible to distinguish two types of fusion proteins. On the one
35 hand fusion proteins which consist of the required protein and a usually short oligopeptide. This oligopeptide ("tag") functions as a marker or recognition sequence for the required protein. A tag may additionally simplify purification.

- 5 -

The main use of the tag is firstly in the testing of expression and secondly in protein purification. One example thereof is the so-called His tag which consists of a peptide sequence which has six consecutive histidine residues and is directly linked to the recombinant protein. With the aid of the attached His residue it is easily possible to purify the fusion protein on a metal affinity column (Smith et al., 1988). This His tag is detected simply with the aid of the highly specific monoclonal antibody His-1 (Pogge v. Strandmann et al., 1995). Another marker used in fusion proteins is GFP, a green fluorescent protein (GFP) which is derived from the jellyfish *Aequorea victoria* and is employed as bioluminescent protein in various biotechnological applications (Kendall and Badminton, 1998; Chalfie et al., 1994; Inouye et al., 1994). It can easily be detected by its autofluorescence in living cells, gels and even live animals.

Further examples of tags, which will not be explained further, are the Strep-tag system (Uhlén et al., 1990) or the myc epitope tag (Pitzurra et al., 1990).

The main use of fusion proteins consisting of a recombinant protein and a **functionally active protein** is, besides the detection described above, in the simplified purification of the expressed fusion proteins. Among these, various systems are known, some of which will be mentioned briefly hereinafter.

In the GST system, fusion vectors make it possible to express complete genes or gene fragments in a fusion with glutathione S-transferase. The GST fusion protein can easily be purified from the cell lysates by affinity chromatography on glutathione-Sepharose (Smith, Johnson, 1988). A biochemical and an immunological detection is available. The maltose-binding protein in the MBP system is a periplasmic protein from E. coli which is involved in transporting

maltose and maltodextrins through the bacterial membrane (Kellermann et al., 1982). It has been used in particular for expressing and purifying alkaline phosphatase on a crosslinked amylose column. The intein system is specifically suitable for rapid purification of a target protein. The intein gene has the sequence for the intein chitin binding domain (CBD), through which the fusion protein can be bound directly from the cell extract onto a chitin column and thus purified (Chong et al., 1997).

Glucose dehydrogenase (GlcDH) is a key enzyme during the early phase of sporulation in *Bacillus megaterium* (Jany et al., 1984). It specifically catalyses the oxidation of β-D-glucose to D-gluconolactone, with NAD^+ or $NADP^+$ acting as coenzyme. Apart from bacterial spores, the enzyme also occurs in the mammalian liver. Two mutually independent glucose dehydrogenase genes (gdh) exist in B. megaterium M1286 (Heilmann et al., 1988). GdhA and gdhB differ considerably in nucleotide sequence, whereas GlcDH-A and GlcDH-B have, despite differences in the protein sequence, approximately the same substrate specificity. Further information and the corresponding DNA and amino acid sequences are also to be found, for example, in EP-B 0290 768.

The systems described above for detecting foreign proteins which have been formed by recombination and whose biological function is either unknown or inadequately known are usually complicated and time-consuming. This means that improvement and optimization of the expression conditions often cannot be done quickly or simply enough.

It is therefore a great advance to have developed a fusion protein partner which makes faster detection of the fusion protein possible, and does not have the disadvantages described in the state of the art for comparable systems.

It has now been found that fusion proteins which comprise GlcDH or a sequence which [lacuna] the biological activity of GlcDH are outstandingly suitable for detecting any required "foreign or target protein" more quickly, simply and thus efficiently than using the state of the art described. This property is based on the surprising finding that GlcDH retains its enzymatic activity under conditions under which other enzymes are inactivated (for example with SDS-PAGE).

The possibility of purifying dehydrogenases on immobilized dyes such as Cibachron Blue 3 G or other NAD-analogous compounds such as aminohexyl-AMP, which are similar, owing to their structure, to the NAD^+ coenzyme and likewise bind to all dehydrogenases, is known.

Thus, as part of a fusion protein, glucose dehydrogenase facilitates, owing to its affinity for the dyes which are, for example, immobilized on a gel and which are commercially available, the purification of the fusion protein in one step. It is furthermore possible to detect GlcDH as constituent of a fusion protein by coupling the enzymatic reaction to a sensitive colour reaction, preferably with iodophenyl-nitrophenyl-phenyltetrazolium salt (INT) or nitro blue tetrazolium salt (NBT) (under the stated conditions), which further simplifies indirect detection of the foreign protein. The method for staining GlcDH as marker enzyme additionally has the advantage that it does not impede the customary staining of proteins using, for example, Coomassie dyes or silver staining in the same gel.

In one embodiment of the present invention, the fusion protein consists of, besides GlcDH and the foreign protein, also a tag peptide which can be used for additional characterization of the proteins bound to the tag peptide. The characterization takes place, for example, via the polyhistidine tag, which is recognized

as antigen by specific antibodies. Detection of the resulting antigen-antibody complex then takes place, for example, using a peroxidase (POD)-labelled antibody via methods known per se. The bound peroxidase

5 produces, after addition of an appropriate substrate (for example ECL system, Western Exposure Chemiluminescent Detection System, from Amersham), a chemiluminescent product which can be detected using a film suitable for this purpose. The immunological

10 detection can, however, also take place by a technique known per se, through a specific antibody tag, for example the myc tag. The polyhistidine tag, alone or in combination with the myc tag, additionally has the advantage that the fusion protein can be purified by

15 binding to a metal chelate column.

However, the GlcDH fusion protein can also be purified and isolated by affinity chromatography directly on a specific anti-GlcDH antibody which has, for example,

20 been immobilized on a chromatography gel such as agarose.

Another advantage of the invention is that GlcDH can be expressed in soluble form in high yields, preferably in

25 E. coli by the known expression systems (see above). Thus, recombinant glucose dehydrogenase from *Bacillus megaterium* M1286 has been successfully expressed with high enzymatic activity in *E. coli* (Heilmann 1988). The expression of other eukaryotic genes in *E. coli* is

30 often limited by the instability of the polypeptide chain in the bacterial host. Incorrect folding may lead to aggregation ("inclusion bodies"), reduced or absent biological activity and proteolytic degradation. A corresponding fusion gene in which the GlcDH gene or a

35 fragment having the biological activity of GlcDH has been ligated to the gene for the required foreign protein, can now be converted according to the invention into the fusion protein with virtually unchanged expression rate and yield compared with the

GlcDH gene without fusion partner. This can also take place when expression of the foreign protein on its own is not possible per se or is possible only in reduced yields or only in an incorrectly folded state or only by use of additional techniques. It is thus possible to obtain the required foreign protein by subsequent elimination of the marker protein GlcDH or of the target protein, for example with endoproteases.

An example according to the invention of a target protein which can be expressed successfully as fusion protein together with GlcDH in E. coli is tridegin. Tridegin is an extremely effective peptide inhibitor for blood coagulation factor XIIIa and is derived from the leech *Haementeria ghilianii* (66 AA, 7.6 kD; Finney et al., 1997).

However, there are no restrictions to be mentioned according to the invention in relation to the nature and the properties of the foreign protein employed.

The invention is not restricted just to the expression of the fusion proteins according to the invention in E. coli. On the contrary, such proteins can also be synthesized advantageously using methods known per se and appropriate stable vector constructs (for example with the aid of the human cytomegalovirus (CMV) promoter) in mammalian, yeast or insect cells with good expression rates.

It is accordingly possible from the above description to characterize the invention in summary as follows and as indicated in the claims:

The invention thus relates to a recombinant fusion protein consisting of at least a first and second amino acid sequence, the first sequence having the biological activity of glucose dehydrogenase. The invention particularly relates to a corresponding recombinant

fusion protein in which the said second sequence is any recombinant protein/polypeptide X or represents parts thereof.

5   The fusion proteins according to the invention may additionally comprise recognition sequences, in particular tag sequences. The invention thus relates further to a corresponding fusion protein which may additionally have at least one other tag sequence or
10 recognition sequence suitable for detection.

The fusion proteins according to the invention have a wide variety of possible uses. In this connection, glucose dehydrogenase with its properties plays the
15 crucial part. Thus, the invention relates to the use of glucose dehydrogenase as detector protein for any recombinant protein/polypeptide X in one of the said fusion proteins. The invention further relates to the use of glucose dehydrogenase in a detection system for
20 the expression of a recombinant protein/polypeptide X as constituent of a corresponding fusion protein. The invention further relates to the use of GlcDH for detecting protein-protein interactions, where one partner corresponds to the recombinant
25 protein/polypeptide X as defined hereinbefore and hereinafter. Finally, GlcDH may serve according to the invention as detector protein for any third protein/polypeptide which is not a constituent of the fusion protein but is able to bind to the second
30 sequence of the protein/polypetide X in the said fusion protein. GlcDH can furthermore be employed as marker protein for a partner in ELISA systems, Western blot and related systems.

35 Since the invention employs recombinant techniques it also, of course, comprises corresponding vectors, host cells and expression systems. The invention relates not only to these vectors and host cells as such but also to the use of corresponding expression vectors in

optimizing the expression of a recombinant protein/polypeptide X in a recombinant preparation process, and to the use of a corresponding host cell in optimizing the expression of a recombinant
5 protein/polypeptide X in such a preparation process.

The invention also relates to a method for the rapid detection of any recombinant protein/polypeptide X by gel electrophoresis, in particular SDS-PAGE gel
10 electrophoresis, where a corresponding fusion protein is prepared and fractionated by gel electrophoresis, and the recombinant protein/polypeptide to be detected is visualized in the gel via the enzymic activity of glucose dehydrogenase.
15

Employed according to the invention in this connection to detect the enzymic activity of glucose dehydrogenase is a colour reaction based on tetrazolium salts, in particular iodophenylnitrophenyl-phenyltetrazolium salt
20 (INT) or nitro blue tetrazolium salt (NBT), it being possible for a general protein staining according to the state of the art to follow [sic] where appropriate before or after the said colour reaction has taken place.
25

The figures are briefly explained below

Fig. 1: Construction scheme for the vector pAW2. The vector contains the sequence for GlcDH. The complete
30 sequence is depicted in Seq. Id. No. 1.

Fig. 2: Construction scheme for the vector pAW3.

Fig. 3: Construction scheme for the vector pAW4. The
35 vector contains the sequence for GlcDH and tridegin. The complete sequence is depicted in Seq. Id. No. 3.

Fig. 4: Staining of GlcDH on an SDS-PAA gel. The staining method is described in detail in the examples.

1: Rainbow marker; **2**: 0.1 µg of GlcDH; **3**: 0.05 µg of GlcDH; **4**: 0.001 µg of GlcDH; **5**: lysate of HC11 cells; **6**: prestained SDS marker.

Fig. 5:   Detection of the expressed GlcDH enzyme (15% SDS-PAA gel, INT stain); **1**: Rainbow marker; **2**: 0.2 µg of native GlcDH; **3**: 10 µl of cell extract/1 ml of clone 2 suspension; **4**: 10 µl of cell extract/1 ml of clone 1 suspension; **5**: prestained SDS marker; cell extract volume: 100 µl.

Fig. 6:   Serial dilutions from pAW2 expression (15% SDS-PAA gel, INT stain); **1**: Rainbow marker; **2**: 10 µl of cell extract/100 µl of suspension; **3**: 10 µl of cell extract/1:5 dilution; **4**: 10 µl of cell extract/1:10 dilution; **5**: 10 µl of cell extract/1:20 dilution; **6**: 0.5 µg of GlcDH; **7**: broad-range SDS marker; **8**: prestained SDS marker; cell extract volume: 100 µl.

Fig. 7:   Detection of the expressed tridegin/GlcDH fusion protein (10% SDS-PAA gel, INT/CBB); **1**: broad-range SDS marker; **2**: 1 µg of GlcDH; **3**: 0.5 µg of GlcDH; **4**: 0.1 µg of GlcDH; **5**: 500 µl of cell extract; **6**:200 µl of cell extract; **7**: 100 µl of cell extract; **8**: 500 µl of cell extract (pAW2 expression); cell extract volume: 100 µl.

Fig. 8:   Immunodetection of tridegin/His and tridegin/His/GlcDH fusion protein (from 10% SDS-PAA gel, ECL detection) and comparison with tridegin/His/GlcDH (10% SDS-PAA gel, INT-CBB stain); **1**: broad-range marker; **2**: 1 ml of cell extract (pAW2 expression); **3**: 100 µl of cell extract (pST106 expression); **4**: 200 µl of cell extract (pST106 expression); **5**: 300 µl of cell extract (pAW4 expression); **6**: 2.5 µg of calin-His positive control; **7**: broad-range marker; **8**: 100 µl [lacuna] (pAW4 expression); cell extract volume: 100 µl.

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<u>Fig. 9:</u> SDS gel which explains the sensitivity of the
detection of GlcDH. 1, 5, 10, 25 and 50 ng of GlcDH and
molecular weight markers (left-hand column) are
plotted.

5

The abbreviations used hereinbefore and hereinafter are
explained below
A   adenine
A_x · absorption at x nm
10   Ab   antibody
     Amp   ampicillin
     AP   alkaline phosphatase
     APS   ammonium peroxodisulphate
     AA   amino acid
15   bla   β-lactamase gene
     BIS   N,N'-methylenebisacrylamide
     bp   base pairs
     BSA   bovine serum albumin
     C   cytosine
20   cDNA   copy (complementary) DNA
     CBB   Coomassie Brilliant Blue
     CIP   calf intestinal phosphatase
     dNTP   2'-deoxyribonuceloside [sic] 5'-triphosphate
     ddNTP   2',3'-deoxyribonuceloside [sic] 5'-triphosphate
25   DMF   dimethylformamide
     DMSO   dimethyl sulfoxide
     DNA   deoxyribonucleic acid
     dsDNA   double-stranded DNA
     DTT   dithiothreitol
30   ECL   Exposure™ Chemiluminescence
     EDTA   ethylenediamine-N,N,N',N'-tetraacetic   acid,
                 disodium salt
     ELISA   enzyme-linked immunosorbent assay
     EtBr   ethidium bromide
35   EtOH   ethanol
     f.c.   final concentration
     FACS   fluorescent-activatet [sic] cell sorting
     G   guanine
     GFP   green fluorescent protein

GlcDH	glucose dehydrogenase (protein)
gdh	glucose dehydrogenase (gene)
GST	glutathione S-transferase
His	histidine residue
HRP	horseradish peroxidase
IB	inclusion body
IgG	immunoglobulin G
INT	iodonitrotetrazolium violet
kb	kilobase pairs
kD	kilodalton
mA	milliampere
m-RNA	messenger RNA
MBP	maltose-binding protein
MCS	multiple cloning site
M_r	relative molecular weight
NAD(P)	nicotinamide adenine dinucleotide (phosphate), free acid
Od_x	optical density at x nm
ompA	outer membrane protein A
ori	origin of replication
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
POD	peroxidase
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulfate
ssDNA	single-stranded DNA
Strep	streptavidin
T	thymine
T_m	melting point (DNA duplex)
t-RNA	transfer RNA
Taq	*Thermophilus [sic] aquaticus*
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine

```
Tet      tetracycline
Tris     tris(hydroxymethyl)aminomethane
U        unit of enzymic activity
U        uracil
5  UV        ultraviolet radiation
ON        overnight
V        volt
VIS       visible
w/v     ·weight per volume
10
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Unless specified otherwise, the methods and techniques used for this invention correspond to methods and processes sufficiently well known and described in the relevant literature. In particular, the disclosure contents of the abovementioned publications and patent applications, especially by Sambrook et al. and Harlow & Lane, and EP-B-0290 768, are comprised in the invention. The plasmids and host cells used according to the invention are as a rule exemplary and can in principle be replaced by vector constructs which are modified or have a different structure, or other host cells as long as they still have the constituents stated to be essential to the invention. The preparation of such vector constructs, and the transfection of appropriate host cells and the expression and purification of the required proteins correspond to standard techniques which are substantially well known and may likewise be modified according to the invention within wide limits.

The invention is described further below. Further details are explained in the examples.

The *Bacillus megaterium* GlcDH structural gene was modified by PCR with the plasmid pJH115 (EP 0290 768) acting as template. The amplified fragment (0.8 kb), which had a PstI recognition sequence at one end and an

Eco47III recogition sequence at the other, was digested with these enzymes and cloned into the cytoplasmic (pRG45) or periplasmic (pST84) *E. coli* expression vector (Figs. 1, 2). The resulting plasmids, pAW2 and pAW3, now had a GlcDH gene which encodes a protein of about 30 kD (261 AA) and is located downstream of the strong Tet promoter. The cytoplasmic pAW2 expression vector has a size of about 4 kb. The periplasmic pAW3 secretion vector is slightly larger and differs from pAW2 only by an omp A signal sequence which is upstream of the multiple cloning site (MCS) and makes it possible for the recombinant protein to be secreted into the periplasm. Both vectors additionally have an MCS with 12 different restriction cleavage sites which make in-frame cloning with the following His tag possible. The polyhistidine (6His) tag makes it possible for the recombinant protein to be purified on a metal affinity column. The vector pAW4 finally comprises the tridegin gene and the GlcDH gene, which were connected together by an MCS, and the polyhistidine (6His) tag which is ligated downstream to the GlcDH gene. The individual constructs are depicted in Figs. 1, 2 and 3. However, the chosen plasmid constructs are only by way of example and do not restrict the invention. They may be replaced by other suitable constructs containing the DNA sequences mentioned. The preparation of the vectors and the clones and expression of the proteins are specified further in the examples.

The sensitivity of the activity staining was carried out [sic] for native GlcDH in a reduced SDS gel. For this purpose, serial concentrations were prepared with native GlcDH (c = 1 mg/ml; A = 200 U/ml), and a negative control was prepared. SDS-PAGE and activity staining using INT resulted in the SDS gel depicted in Fig. 3. It was possible with the test employed to detect GlcDH down to a concentration of 50 ng. The

negative control, which contains no GlcDH, shows no
band, as expected.

The exact molecular weight of the native GlcDH can be
5 determined using marker proteins and with the aid of a
calibration plot. To do this, the relative migration
distances of the marker proteins were determined and
plotted against their respective logarithmic molecular
weights.
10 A procedure for the expressions carried out was as
depicted in the scheme (Tab. 1):

Tab. 1

Transformation of the GlcDH expression vectors into W3110 cells
↓
Preculture in LB(Amp) medium at 37°C (12 h)
↓
Cell growth at 37°C in main culture with induction (5 h)
↓
Centrifugation to obtain biomass
↓
Suspension of the cells in 1x SDS loading buffer
↓
Cell disruption at 95°C for 5 min
↓
Cell extract can be used directly in SDS-PAGE (1 h)
↓
Activity staining of GlcDH in SDS gel (30 min)
↓
Gel band analysis

15 The plasmid pAW2/clone 9 (pAW2/K9) was transformed into
the competent *E. coli* expression strain W3110, and two
clones from the resulting transformation plate were
used to inoculate a 5 ml preculture. Induction with
anhydrotetracycline took place 2 h after inoculation of
20 the main culture. Expression overall lasted 5 h and was
stopped at an OD of 1.65 for clone 1 and 1.63 for clone

2. After SDS-PAGE and GlcDH activity staining, a strong
GlcDH band (about 35 kD) was detectable for each clone
from 1 ml of cell suspension.

No difference between the resulting GlcDH bands became
5   evident when SDS-PAGE was carried out under reduced and
non-reduced conditions. For this purpose, in each case
500 to 100 μl of the cell suspension were investigated
in the SDS gel by GlcDH activity staining with INT.

10 In order to illustrate the sensitivity of the GlcDH
activity staining compared with Coomassie staining,
samples of 100 μl of cell suspension, and 1/5, 1/10 and
1/20 dilutions of the cell suspension were prepared.
The final volume of the dilutions was likewise 100 μl.
15 The resulting SDS gel was used, after the GlcDH
activity staining, for a Coomassie staining to
visualize further protein bands. The SDS gel resulting
from this is depicted in Figure 4. A distinct band is
still evident at the 1/20 dilution using the GlcDH
20 activity staining, whereas Coomassie-stained bands are
now scarcely detectable.

The *Haementeria ghilianii* tridegin structural gene with
coupled His tag was modified by PCR with the plasmid
25 pST106 acting as template. The amplified fragment
(0.25 kb), which is flanked by a ClaI recognition
sequence and a PstI recognition sequence, was digested
with these enzymes and cloned into the cytoplasmic *E.
coli* GlcDH fusion vector pAW2. The resulting plasmid
30 pAW4 now had a tridegin-His-GlcDH fusion protein gene
which codes for a protein of about 44 kD and is located
downstream of the strong Tet promoter. The cell extract
from the *E. coli* strain W 3110 which comprises the
cytoplasmic pAW4 plasmid was analysed by SDS-PAGE and
35 GlcDH activity staining. It was possible therewith to
detect several bands stained red-violet at 35, 37, 40
and 43 kD. The 43 kD band comprised the required
tridegin-His-GlcDH fusion protein, although its
molecular weight was somewhat less than the theoretical

value of 44 kD. The remaining detectable bands were presumably produced by proteolytic degradation of the fusion protein in *E. coli* since the smallest stained band of 35 kD approximately corresponds to the size of GlcDH. It was possible on the basis of a size comparison to identify the 35 kD band which was formed as the His-GlcDH degradation product.

Carrying out [sic] expression kinetics revealed that proteolytic degradation of the formed fusion protein started 2 hours after induction of the Tet promoter with anhydrotetracycline, that is to say after this time additional bands were detectable in the SDS gel by activity staining. The formed fusion protein was not stable to *E. coli* proteases, which is shown by its relatively fast protein degradation. It was possible, by using the constructed periplasmic GlcDH fusion vector pAW3 to avoid proteolytic degradation of the fusion protein in the cell, because in this case the expressed fusion protein would be secreted into the periplasmic space between *E. coli* cells. *E. Coli* proteases are found mainly in the cytoplasm.

The sensitivity and specificity of the GlcDH fusion protein detection makes it possible for recombinant foreign proteins to be screened rapidly and simply. Sensitivity of the GlcDH detection system was determined using native GlcDH. Detection of native GlcDH activity resulted in a band stained red-violet at about 30-35 kD in the SDS-PAA gel.

Cytoplasmic expression in the E. coli strain W 3110 of the recombinant GlcDH from pAW2 showed the same molecular weight. Sensitivity comparison between native GlcDH and recombinant GlcDH was possible by comparing the band intensities.

The developed test system (see examples) additionally makes it possible to carry out double staining of the SDS gels. In the first staining there is specific detection of the GlcDH bands. The background staining can be followed by a conventional protein staining, for

example a Coomassie staining of the remaining proteins. GlcDH surprisingly retains according to the invention under reducing conditions in the presence of SDS its complete activity, which makes rapid detection in the
5   SDS gel possible.

It is furthermore possible according to the invention to increase the sensitivity of the detection of GlcDH activity by using nitro blue tetrazolium salt (NBT) as
10 substrate for GlcDH. The reaction rate for the GlcDH detection using INT can, however, be increased further by using Triton X-100 (1% final solution) or adding NaCl (1 M final solution).

15 The recombinant fusion proteins tridegin/His and tridegin/His/GlcDH were obtained by expression of the pST106 and pAW4 plasmids (Figs. 1, 2). After disruption of the cells in the relevant expression mixture, the samples were fractionated by SDS-PAGE and transferred
20 to a membrane. The tridegin-His-GlcDH fusion protein was detectable immunologically via the His tag present therein by using an anti-[RGS]His antibody in a Western blot. The controls used were purified recombinant calin (leech protein) which has a terminal His tag, and the
25 cell extract of the expressed recombinant GlcDH which has no His tag. The anti-[RGS]His antibody was able to detect a band at about 37 kD and another band at about 43 kD for the recombinant tridegin/His/GlcDH fusion protein (Fig. 6). Comparison of the sizes of the bands
30 obtained with the bands obtained after activity staining in the SDS gel shows that the 43 kD band represents the tridegin-His-GlcDH fusion protein and the 37 kD band represents the His-GlcDH degradation product of the complete fusion protein. The calin/His
35 tag protein produced a band at about 26 kD. The somewhat smaller recombinant tridegin/His tag protein produced a band at about 23 kD plus further bands indicating binding of the His antibody to other expressed proteins. The immunological detection with

the anti-[RGS]His antibody thus proves that the protein
detected at 43 kD and that detected at 37 kD contained
a His tag. In addition, the size of the latter protein
approximately corresponded to the theoretical size
(36.5 kD) of the GlcDH protein with coupled His tag.

In addition to the detection of expression of the
recombinant tridegin, the biological activity of
tridegin as constituent of the tridegin-GlcDH fusion
protein was investigated, in the specific case from
pAW4. This test is based on the inhibition of factor
XIIIa by native leech gland homogenate and purified
tridegin (Finney et al., 1997). The modified test is
described in the examples. As a control, the
corresponding fusion protein from pST106 and the GlcDH
protein from pAW2 were expressed. Comparison of the
enzymic activity with recombinant tridegin expressed
either as GlcDH-tridegin fusion protein or as tridegin-
His tag in E. coli revealed negligible differences. In
addition, the recombinant tridegin proteins from the
two different expressions showed comparable biological
activities to the native leech gland homogenate. It can
be concluded from this that fusion with GlcDH has no
interfering effect at all on the biological activity of
the coexpressed foreign gene.

Tridegin itself (that is to say not as fusion protein)
has no activity after E. coli expression and is formed
as inclusion body. Expression of GlcDH in E. coli
results in an enzyme with high specific activity and
stability in soluble form. It was demonstrated in
expression experiments that proteins which have a high
solubility capacity on expression in E. coli increase
the solubility capacity of foreign protein expression
when they are fused to the latter (LaVallie, 1995).
Fusion of tridegin to GlcDH in this case also increased
the solubility of tridegin because it was possible by a
biological detection in which tridegin inhibits factor
XIIIa to detect the activity of tridegin after E. coli

expression as tridegin-His-GlcDH fusion protein. The GlcDH fusion protein is expressed in high yield in *E. coli*.

The possibility of expressing cloned genes as fusion
5   proteins containing a protein of known size and biological function markedly simplifies the detection of the gene product. For this reason, as mentioned in the introduction, numerous fusion expression systems have been developed with various detection strategies.
10

A comparison of the known systems with the GlcDH fusion system according to the invention in E. coli is shown in Tab. 2. In some systems, the N-terminal fusion protein can be cleaved off from the C-terminal target
15 or foreign protein (Collins-Racie et al., 1995).

Tab. 2:

Tag/fusion partner	MW (kD)	Detection	Advantage
GlcDH	30	Function test in the SDS gel	Rapid and low-cost, direct detection in the SDS gel
His tag (Pogge v. Strandmann et al., 1995)	1-7	Western blot, ELISA	Small
Strep-tag (Uhlén et al., 1990)	13	Western blot,	Small
myc epitope (Pitzurra et al., 1990; Gazitt et al., 1992)	1-2	Western blot, ELISA	Small
IgG portions, Fc (Moks et al., 1987; Ettinger et al., 1996)	2-5	Western blot, ELISA	Small, selection of cells (FACS)
GFP (Chalfie et	27	Fluorescence,	Selection of

al., 1994; Inouye et al., 1994)		Western blot	cells even in the culture dish, several detectable simultaneously (FACS)
Intein (Chong et al., 1997)	48	Western blot	Fusion partner can be deleted
GST (Smith, Johnson, 1988; Gosh et al., 1995)	26	Western blot, colorimetric detection in solution	Fusion partner can be deleted
MBP (Chu di Guan et al., 1988; Kellermann et al., 1982)	40	Western blot	Fusion partner can be deleted

Method	Pre-condition	Time taken	Throughput	Sensitivity	Information
GlcDH detection	GlcDH functionally active	about 3 h	moderate-high	50 ng	protein amount + protein size
ELISA	2 antibodies	about 1 day	high	pg-ng	protein amount
Western blot	1-2 antibodies Tag on the protein	1-2 days	low	ng	protein size + protein amount

A very great advantage of the GlcDH detection system according to the invention is the fact that it does not require, such as, for example, for the Western blot detection, any antibodies or other materials such as, for example, membranes, blot apparatus, developer machine with films, microtitre plates, titre plate reader etc. This means that the detection of

recombinant fusion proteins using the GlcDH system takes place very much more favourably and rapidly. It is possible with the aid of GlcDH detection to establish not only information about the amount of the expressed fusion protein but also the corresponding size of the fusion protein directly in the SDS-PAA gel without transfer to a membrane. If GlcDH activity is detectable in the fusion protein, the fusion partner ought also as a rule to be functionally active. GlcDH does not interfere with the folding of the fusion partner. The advantages of the GlcDH fusion protein system according to the invention are shown in a comparison hereinafter (Tab. 3 below) by selecting from the literature an efficient method for isolating and detecting a fusion protein obtained in *E. coli*.

The GlcDH fusion protein system according to the invention is furthermore particularly suitable for increasing the solubility of proteins which are formed, especially in *E. coli*, as inclusion bodys and therefore make subsequent protein purification difficult and costly. It is normally necessary to convert proteins formed as inclusion bodys into their native state by elaborate methods. This is unnecessary on use of the fusion proteins according to the invention.

In summary, the advantages of the fusion proteins according to the invention which are in use as GlcDH detection system are as follows.

- Stability under SDS and reducing (denaturing) conditions
- Sensitive GlcDH-specific enzymatic colour test
- Sensitivity as far as at least 50 ng
- Rapid detection directly in the SDS gel with determination of the molecular weight of the fusion partner
- Possibility of additional protein stainings
- Low-cost materials, little expenditure on apparatus

- Good expression in E. coli, including that of the target protein with retention of the biological activity
- Possibility of avoiding inclusion bodies of the foreign/target protein or other aggregates produced by incorrect folding
- Possibility of purifying the fusion protein via affinity chromatography, for example on dyes (Cibacron Blue 3G)

Tab. 3

Construction/transformation of the <u>protein A/GFP fusion vector</u>	Construction/transformation of the <u>GlcDH/tridegin fusion vector</u>
↓	↓
Growth of the cells on LB agar plates at 37°C (1 day)	Preculture in LB(Amp) medium at 37°C (12 h)
↓	↓
Cell growth at 25°C (3 days)	Cell growth at 37°C in main culture with induction (5 h)
↓	↓
Suspension of the cells In buffer (pH 8.0)	Suspension of the cells in SDS loading buffer
↓	↓
Cell disruption and removal of cell detritus by centrifugation	SDS cell disruption at 95°C for 5 min
↓	↓
SDS-PAGE for protein separation (1 h)	SDS-PAGE (1 h) with cell extract
↓	↓
Protein transfer to nitrocellulose membrane (1 h)	
↓	
Blocking reaction (1 h)	GlcDH activity staining in

↓ Antibody reaction (1 h) ↓ Incubation in protein A-GFP working buffer ·(20 min) ↓ UV radiation (365 nm)/analysis of the blot	the SDS gel (30 min) ↓ Analysis of the SDS gel with determination of the molecular weight

The following examples illustrate the invention further without restricting it.

5   **Example 1:**

Primer	Sequence	Length	Use
GlcDH#1	5'- GCGCGAATTCATGTATA CAGATTTAAAAAGAT- 3'	32 bases	PCR primer (attaches to the 5' end of gdh and introduces an EcoRI cleavage site)
GlcDH#2	5'- GCGCTTCGAACTATTAG CCTCTTCCTGCTTG-3'	31 bases	PCR primer (attaches to the 3' end of gdh and introduces an SfuI cleavage site)
GlcDH#3	5'- GCGCCTGCAGATGTATA CAGATTTAAAAGAT-3'	31 bases	PCR primer (attaches to the 5' end of gdh and introduces a PstI cleavage site)
GlcDH#4	5'- GCGCAGCGCTCTATTAG CCTCTTCCTGCTTG-3'	31 bases	PCR primer (attaches to the 3' end of gdh and introduces an Eco47III cleavage site)

Tridegin #1	5'-GCGCATCGATATGAAACTATTGCCTTGCAAA-3'	31 bases	PCR primer (attaches to the 5' end of tridegin and introduces a ClaI cleavage site)
Tridegin #2	5'-GCGCCTGCAGGTGATGGTGATGGTGATGCGA-3'	31 bases	PCR primer (attaches to the 3' end of tridegin and introduces a PstI cleavage site)
pASK 75 UPN	5'-CCATCGAATGGCCAGATGATTA-3'	22 bases	Sequencing primer (IRD 41 labelled at the 5' end, attaches in tet p/o of pRG 45 and pST84)
PASK 75 RPN	5'-TAGCGGTAAACGGCAGACAAA-3'	21 bases	Sequencing primer (5' IRD 41 labelled, attaches in lpp of pRG 45 and pST84)
T7 Seq.s	5'-TAATACGACTCACTATAGGG-3'	20 bases	Sequencing primer (5'IRD 41 labelled, attaches to the T7 priming site of pcDNA3.1/myc-His A, B, C
Rev Seq.as	5'-TAGAAGGCACAGTCGAGG-3'	18 bases	Sequencing primer (5' IRD 41 labelled, attaches to the BGH reverse priming site of pcDNA3.1/myc-His A, B, C)

The above nucleotides were used according to the invention (Tab. 4).

5   Table 5 below summarizes the microorganisms used. All the microorganisms are derived from *E. coli* K12 and belong to risk group 1.

Tab. 5

Strain	Genus/ species	Genotype	Literature
Top10F' One Shot™ Cells	*E. coli*	F'(*lacI*�q*Tn*10(Tet^R))*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC)Φ80*lacZ*ΔM15Δ*lacX*74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(StrR) *end*A1 *nup*G	Top10F' OneShot™ Kit from Invitrogen®
Epicurian Coli®XL1- Blue MRF' Cells	*E. coli*	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 *thi-1* *rec*A1 *gyr*A96 *rel*A1 *lac*(F' *pro*AB *lacI*ᵍZΔM15Tn10(Tet^I))	Stratagene's Competent Cells
TOP10 OneShot™ Cells	*E. coli*	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lacX*74 *rec*A1 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	TOPO TA Cloning® Kit (Version C) from Invitrogen®
W 3110	*E. coli*	F⁻ λ⁻ WT *E. coli*	B. Bachmann, Bacteriol. Rev. 36(72) 525-557

Donor organism: M 7037 expression strain (E. coli N 4830/pJH 115) of 21.10.96 (supplied by Merck).

pJH 115: pUC derivative, 5.9 kb, O_LP_L promoter, gdh, to (terminator), galk (galactosidase gene), bla (β-lactamase gene), ori (origin of replication), 2 HindIII, 2 BamHI and one each EcoRI and ClaI cleavage site.

Example 2:

Transformation of plasmids into competent E. coli cells:

<u>SOC medium:</u> 20 g of Bacto tryptone, 5 g of Bacto yeast extract, 0.5 g of NaCl, 0.2 g of KCl ad 1 l ddH$_2$O, autoclave. Before use, add: 0.5 ml of 1 M MgCl$_2$/1 M MgSO$_4$ (sterile-filtered), 1 ml of 1 M glucose (sterile-filtered)

<u>LB(Amp) agar plates:</u> mix together 1 l of LB medium (without ampicillin) and 15 g of agar-agar, autoclave, cool to about 60°C and 1 ml of ampicillin solution (100 mg/ml). Procedure:

<u>Mixture</u>   1-5 μl   of ligation product or plasmid
                                DNA (5-50 ng/μl)

              50 μl   of competent cells

              450 μl   of SOC medium

- thaw competent cells on ice for 10 min
- add DNA to the competent cells
- incubate on ice for 30 min
- heat shock: 30 sec at 42°C (water bath)
- place cells on ice for 2 min
- add 450 μl of prewarmed SOC medium
- incubate at 37°C and 220 rpm for 1 h
- streak 100 μl portions of the mixture onto a prewarmed LB(Amp) plate
- incubate plates at 37°C overnight

Example 3:

TOPO-TA-Cloning® and ligation

TOPO-TA-Cloning® is a five-minute cloning method for PCR products amplified with Taq polymerase.

The TOPO-TA-Cloning® kit (version C) supplied by Invitrogen was developed for direct cloning of PCR products. The system makes use of the property of thermostable polymerases which attach a single deoxyadenosine at the 3' end of all duplex molecules in a PCR (3'-A overhang). It is possible with the aid of these 3'-A overhangs to link the PCR products directly to a vector which has 3'-T overhangs. The kit provides the pCR®2.1-TOPO vector which was specifically developed for this purpose. The vector is 3.9 kb in size and has a *lacZ* gene for blue/white selection, and ampicillin- and kanamycin-resistant genes. The cloning

site is flanked on both sides by a single EcoRI
cleavage site.

Ligation mixture:

2 μl   of fresh PCR product (10 ng/μl)

5   1 μl   of pCR®-TOPO vector

.2 μl   of sterile water

5 μl   total volume

.   Carefully mix the mixture and incubate at RT for 5
    min

10   .   Briefly centrifuge and place tube on ice

    .   Employ ligation products immediately in the One
    Shot™ transformation

A 5 μl mixture without PCR product and consisting only

15   of vector and water is used as control.

The One-Shot™ transformation was carried out by the
following method:

Add 2 μl of 0.5 M β-mercaptoethanol to the 50 μl of One
Shot™ TOP10 competent cells thawed on ice;

20   Add 2 μl of the TOPO-TA-Cloning® ligation per vial of
competent cells;

Incubate on ice for 30 min

Heat shock: 30 sec at 42°C;

Cool on ice for 2 min;

25   Add 250 μl of SOC medium (RT);

Incubate the vials at 37°C and 220 rpm for 30 min;

Streak 100 μl of each transformation mixture onto
LB(Amp) plates prewarmed to 37°C;

Incubate plates at 37°C overnight;

30   Analyse   the   resulting   transformands   after
minipreparation (3.2.2.1) with suitable enzymes in an
analytical restriction digestion.

Example 4:

35   *Gene expression in E. coli cells*

The procedure is outlined as follows:

    .   The plasmid is isolated from successfully
sequenced clones and transformed into the expression
strain W3110

. A clone is picked from the transformation plate and used to prepare a 5 ml ON preculture

. The preculture is streaked onto an LB(Amp) plate, and clones from this plate are used to inoculate expressions to be carried out later

. 1 ml of the preculture is then used to inoculate the 50 ml main culture (ratio 1:50) and the OD_{600} is determined (reference measurement with uninoculated LB(Amp) medium)

. The main culture (in a 200 ml Erlenmeyer flask) is incubated at 37°C and 220 rpm

. The OD_{600} is determined every 30 min

. Once the OD reaches 0.5, the cells are induced with 10 μl of anhydrotetracycline (1 mg/ml) per 50 ml of cell suspension (f.c. 0.2 μg of anhydrotetracycline per ml of cell suspension), and the OD is again determined (0 value)

. The OD is determined every hour and growth is stopped 3 h after the time of induction

. 1 ml of thoroughly mixed bacterial suspension is placed in a tube and centrifuged at 6000 rpm for 5 min (less suspension may also be used if necessary)

. The supernatant is aspirated off and the pellet is homogenized in 100 μl of 1 x red. sample buffer;

. The homogenate is boiled for 5 min, cooled on ice and briefly centrifuged;

. 10 μl of sample are loaded into each well of an SDS gel and the electrophoresis (3.2.16) is carried out;

. The gel is stained by Coomassie blue staining and/or by the method of Example 5.

Cell disruption:

Cells from a 50 ml overnight culture are centrifuged at 3500 rpm and 4°C for 15 min. The resulting supernatant is poured away and the cells are resuspended in 40 ml of 100 mM Tris/HCl (pH 8.5). The suspended cells are disrupted using a French press in a 1 inch cylinder under 18,000 psi. This entails the cells being forced through a narrow orifice (< 1 mm) and subjected to a

sudden fall in pressure. The cells burst due to the pressure difference on passing through the orifice. The structure of the cellular proteins is retained during this. To avoid proteolytic degradation of the required protein, a protease inhibitor should be added immediately after the cell disruption. For this purpose, 1 tablet of the EDTA-free Complete™ Protease-Inhibitor Cocktail (Roche) is added to each 40 ml of protein solution and dissolved at RT. The subsequent centrifugation at 6000 rpm for 20 minutes removes the cell detritus and large parts of DNA and RNA. The samples are then frozen at -20°C.

Example 5:

Activity staining of the GlcDH band in the SDS gel:
The glucose dehydrogenase band can be specifically detected in the SDS gel using iodophenylnitrophenyl-phenyltetrazolium chloride (INT). This is possible only because the SDS treatment does not destroy the GlcDH activity.
The GlcDH is detected by means of a colour reaction. This entails the hydrogen formed in the reaction being transferred to the tetrazolium salt INT, producing a violet formazan. Phenanzine methosulfate serves as electron transfer agent.

Preincubation buffer (0.1 M Tris/HCl, pH 7.5)
15.76 g   of   Tris/HCl
ad 1 l ddH$_2$O, pH 7.5 with NaOH

Reaction   buffer   (0.08%   INT,   0.005%   phenanzine methosulfate, 0.065% NAD, 5% Glc in 0.1 M Tris/HCl (pH 7.5)
0.8 g   of   iodophenylnitrophenyltetrazolium   chloride (INT)
0.05 g   of   methylphenazinium methosulfate (phenanzine methosulfate)
0.65 g   of   NAD
50 g   of   D-(+)-glucose monohydrate (Glc)

ad 1 1 0.1 M Tris/HCl (pH 7.5)

Storage buffer for GlcDH:

26.5 g of EDTA

15 g of Na$_2$HPO$_4$

ad 1 l, pH 7.0 (NaOH)

Sample preparation:

.   Dilute samples and markers in sample buffer.

.   Boil in a water bath for 3 min and cool on ice, and centrifuge.

SDS gel electrophoresis by standard methods.

Activity staining:

.   Incubate SDS gel with fractionated protein bands in preincubation buffer at 37°C with gentle shaking for 5 min

.   Pour off buffer and cover with a sufficient amount of reaction buffer (RT), and incubate at 37°C with gentle shaking (change buffer at least 1 x)

.   After incubation for about 30 min, the bands with GlcDH are stained red-violet.

.   Wash gel in preincubation buffer, photograph and dry

.   If required, carry out a subsequent Coomassie staining and then dry the gel.

Example 6:

Immunological detection using the ECL system (Western Exposure^{TM} Chemiluminescent Detection System):

Proteins coupled to a His tag are detected indirectly using two antibodies. The first Ab employed is the anti-^{RGS}His antibody (QIAGEN) for detecting 6xHis-tagged proteins. The resulting antigen-antibody complex is then detected using the peroxidase (POD)-labelled AffiniPure goat anti-mouse IgG (H+L) antibody. After addition of the ECL substrate mixture, the bound

peroxidase results in a chemiluminescent product which can be detected using a high performance chemiluminescence film.

Ponceau S solution (0.5% Ponceau S, 7.5% TCA)

5   1.25 g of Ponceau S

18.75 g of TCA

Make up to 250 ml with double-distilled water.

10x PBS buffer pH 7.4

10   14.98 g of disodium hydrogen phosphate x 2 H_2O

2.13 g of potassium dihydrogen phosphate

87.66 g of sodium chloride

Make up to 1 l, check that pH is 7.4.

The 1x concentration of the buffer is employed.

Biometra blot buffer

25 mM   Tris

150 mM   Glycine

10%   Methanol

Blocking reagent

5%   Skimmed milk powder

Dissolve in 1x PBS buffer.

Washing buffer

0.1%   Nonidet™ P-40 (Sigma)

Dissolve in 1x PBS buffer

The detection was carried out as follows:

.   Cut a PVDF membrane (Immobilon P, Millipore) and 6x blotting filter paper to the size of the gel

.   Equilibrate the PVDF membrane for 15 sec in methanol and then in Biometra blot buffer, and apply the same procedure to the SDS gel and the filter papers

.   Blot construction: assemble 3 layers of filter paper, membrane, gel, 3 layers of filter paper in the blot chamber (air bubbles between the layers must be expelled otherwise no protein transfer takes place at these points)

.   Blotting: 1-1.5 mA/cm^2 of gel for 1 h

Check of protein transfer:

After the blotting, the protein transfer to the PVDF membrane is checked by staining with Ponceau S: incubate the membrane with 0.5% Ponceau S solution in a dish with gentle shaking for at least 2 min. Pour off dye (reusable) and destain the membrane under running deionized water. In this case, only strong protein bands are stained. The molecular weight marker is marked with a ballpoint pen.

Development of the blot:

All incubations should be carried out in a dish on a Celloshaker and in a roller cabinet in 50 ml Falcon tubes since the membrane must never dry out during the following steps.

(1) Saturation

30 min at 37°C in a roller cabinet with PBS/5% skimmed milk powder

(2) 1st antibody: incubate diluted 1:2000 in PBS/5% skimmed milk powder (volume about 7 ml/membrane) at 37°C for 1 h

(3) Washing: Wash membrane copiously with washing solution PBS/0.1% NP-40 wash for 3 x 5 min

(4) POD-labelled Ab: incubate diluted 1:1000 in PBS/5% skimmed milk powder (new tube) at 37°C for 1 h

(5) Washing: Wash membrane copiously with washing solution PBS/0.1% NP-40 wash for 3 x 5 min

(6) Development: Swirl membrane thoroughly (do not allow to dry) and place on a plastic sheet, cover completely with ECL developer solution (Amersham) for 1 min, swirl membrane and place in a doubled sheet, lay polaroid Hyperfilm on top and develop

Example 7:

Tridegin detection by inhibition of factor XIIIa (Method of Finney et al., 1997, modified according to the invention):

In place of the natural substrate of factor XIIIa, namely amino-containing side chains of amino acids, synthetic amines are also incorporated into suitable

protein substrates. These synthetic amines have intramolecular markers which make detection possible. The amine incorporation test is a solid-phase test.

The titre plates are coated with casein. The substrate biotinamidopentylamine is incorporated into this casein by factor XIIIa. The casein-biotinamidopentylamine product can be detected by the streptavidin-alkaline phosphatase fusion protein (strep/AP). This sandwich can take place [sic] by detecting the phosphatase activity using p-nitrophenyl phosphate. This involves the following reaction:

$$\text{4-Nitrophenyl phosphate} + H_2O \xrightarrow{\underline{AP}} \text{phosphate} + \text{4-nitrophenolate [sic]}$$
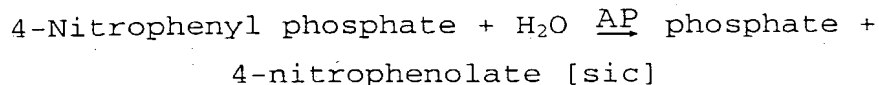
The formation of 4-nitrophenolate [sic] is determined by photometry at 405 nm and is directly proportional to the AP activity. The high-affinity interaction of biotin and streptavidin means that the phosphatase activity is likewise proportional to the factor XIIIa activity, that is to say a stronger absorption (yellow coloration) means a higher factor XIIIa activity (Janowski, 1997). EDTA is a very nonspecific inhibitor of factor XIIIa, whose cofactor Ca^{2+} is bound by EDTA in a chelate complex. For this reason, the protein samples used must not contain any EDTA and were pretreated with an EDTA-free protease inhibitor cocktail (Boehringer).

Washing buffer:	100 mM Tris/HCl, pH 8.5
Solution A:	Dissolve 0.5% skimmed milk powder in washing buffer
Solution B:	Dissolve 0.5 mM biotin-amidopentylamine, 10 mM DTT, 5 mM $CaCl_2$ in washing buffer
Solution C:	Dissolve 200 mM EDTA in washing buffer
Solution D:	Dissolve 1.7 μg/ml of streptavidin-alkaline phosphatase in solution A

Solution E: Dissolve 0.01% (w/v) Triton X-100 in washing buffer

Solution F: Dissolve 1 mg/ml p-nitrophenyl phosphate, 5mM $MgCl_2$ in washing buffer

Coating:
.   Distribute 200 µl of solution A in each well on a titre plate, depending on the number of samples
Shake at 37°C for 30 min (Thermoshaker)

Washing:
.   Wash twice with 300 µl of washing buffer per well

Incorporation reaction:
.   Distribute 10-150 µl of sample per well and add 5 µl of factor XIIIa per well and 200 µl of solution B per well
Shake at 37°C for 30 min

Stopping:
.   Wash twice with 300 µl of solution C (factor XIIIa inhibition) per well
.   Wash twice with 300 µl of washing buffer per well

Strep/Ap binding (specific):
.   Add 250 µl of solution D per well
.   Incubate at RT for 60 min

Washing:
.   Wash with 300 µl of solution E per well (detaches the proteins which are not covalently bonded)
.   Wash 4 times with 300 µl of washing buffer per well

Substrate:
.   Add 50 µl of solution F per well + 200 µl of washing buffer per well
.   Incubate at RT for 30 min
Measure with computer-assisted evaluation in a microtitre plate reader at 405 nm

EXAMPLE 8: Sensitivity of GlcDH detection
The stated amount of purified GlcDH was put on an SDS gel. After the run, the SDS gel was incubated in preincubatiuon buffer at 37°C for 5 minutes. The buffer

was discarded and the gel was shaken in reaction buffer at 37°C. In a further step the gel was stained with Coomassie blue.

Reaction buffer for 1 litre:

5   0.1M Tris/HCL, pH 7.5

0.5M NaCl

0.2% Triton X-100

0.8 g of iodophenylnitrophenyltetrazolium chloride

0.05 g of methylphenazinium methosulfate

10   0.65 g of NAD

50 g of D-(+)-glucose monohydrate

Preincubation buffer:

0.1M Tris/HCl, pH 7.5

0.5M NaCl

Patent claims

1.   Recombinant fusion protein consisting of at least
     a first and second amino acid sequence,
     characterized in that the first sequence has the
     biological activity of glucose dehydrogenase.

2.   Recombinant fusion protein according to Claim 1,
     characterized in that the second sequence is any
     recombinant protein/polypeptide X or represents
     parts thereof.

3.   Recombinant fusion protein according to Claim 2,
     characterized in that it may additionally have at
     least one other recognition sequence ("tag
     sequence") suitable for detection.

4.   DNA, characterized in that it codes for a fusion
     protein according to Claims 1-3.

5.   Expression vector, characterized in that it
     comprises a DNA according to Claim 4.

6.   Host   cell   for   expressing   recombinant
     proteins/polypeptides, characterized in that it
     comprises an expression vector according to Claim
     5.

7.   Use of glucose dehydrogenase as detector protein
     for any recombinant protein/polypeptide X in a
     fusion protein according to Claims 1 to 3.

8.   Use of glucose dehydrogenase in a detection system
     for   the   expression   of   a   recombinant
     protein/polypeptide X as constituent of a fusion
     protein according to Claims 1 to 3.

9. Use of glucose dehydrogenase for detecting protein-protein interactions, where one partner corresponds to the recombinant protein/polypeptide X in Claims 1 to 3.

10. Use of glucose dehydrogenase in a fusion protein according to Claims 1-3 as detector protein for any third protein/polypeptide which is not a constituent of the fusion protein according to Claims 1-3 and is able to bind to the second sequence of the protein/polypetide X in the said fusion protein.

11. Use of an expression vector according to Claim 5 in optimizing the expression of a recombinant protein/polypeptide X in a recombinant preparation process.

12. Use of a host cell according to Claim 6 in optimizing the expression of a recombinant protein/polypeptide X in a recombinant preparation process.

13. Method for the rapid detection of any recombinant protein/polypeptide X by gellectrophoresis, characterized in that a fusion protein according to Claims 1 to 4 is prepared and fractionated by gel electrophoresis, and the recombinant protein/polypeptide to be detected in the gel is visualized via the enzymic activity of glucose dehydrogenase.

14. Method according to Claim 13, characterized in that SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used as gel electrophoresis method.

15. Method according to Claim 13, characterized in that a colour reaction based on tetrazolium salts

is employed to detect the enzymic activity of glucose dehydrogenase.

16. Method according to Claim 15, characterized in that iodophenylnitrophenyl-phenyltetrazolium salt (INT) or nitro blue tetrazolium salt (NBT) is employed as tetrazolium salt.

17. Method according to Claims 13 to 16, characterized in that the specific staining of the glucose dehydrogenase is followed by a general protein staining.
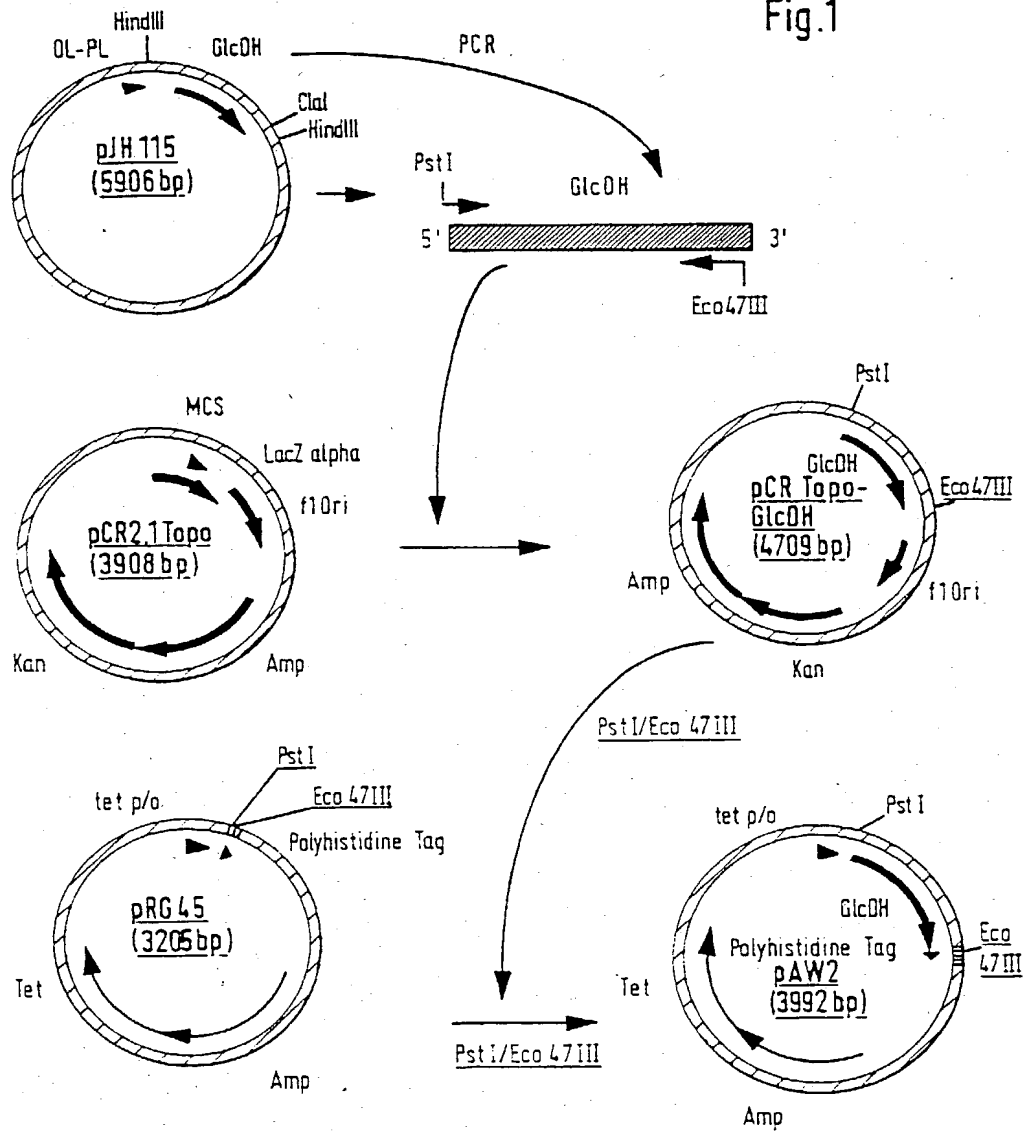
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(54) Title: GLUCOSE DEHYDROGENASE FUSION PROTEINS AND THEIR UTILIZATION IN EXPRESSION SYSTEMS

(54) Bezeichnung: GLUCOSE–DEHYDROGENASE–FUSIONSPROTEINE UND IHRE VERWENDUNG IN EXPRESSIONSSYSTE-MEN

(57) Abstract

The invention relates to novel recombinant fusion proteins containing a protein sequence having the biological activity of glucose dehydrogenase as one of its constituents and to their utilization for simple and efficient detection of any type of proteins/polypeptides in SDS–Page gels and for quick optimization of expression systems that can express the above–mentioned proteins/polypeptides.

(57) Zusammenfassung

Die Erfindung betrifft neue rekombinante Fusionsproteine, welche als ein Bestandteil eine Proteinsequenz mit der biologischen Aktivität von Glucose–Dehydrogenase enthalten sowie ihre Verwendung zum einfachen und effizienten Nachweis von beliebigen Proteinen/Polypeptiden in SDS–PAGE–Gelen und zur raschen Optimierung von Expressionsystemen, welche besagte Proteine/Polypeptide zu exprimieren in der Lage sind.

Fig.1
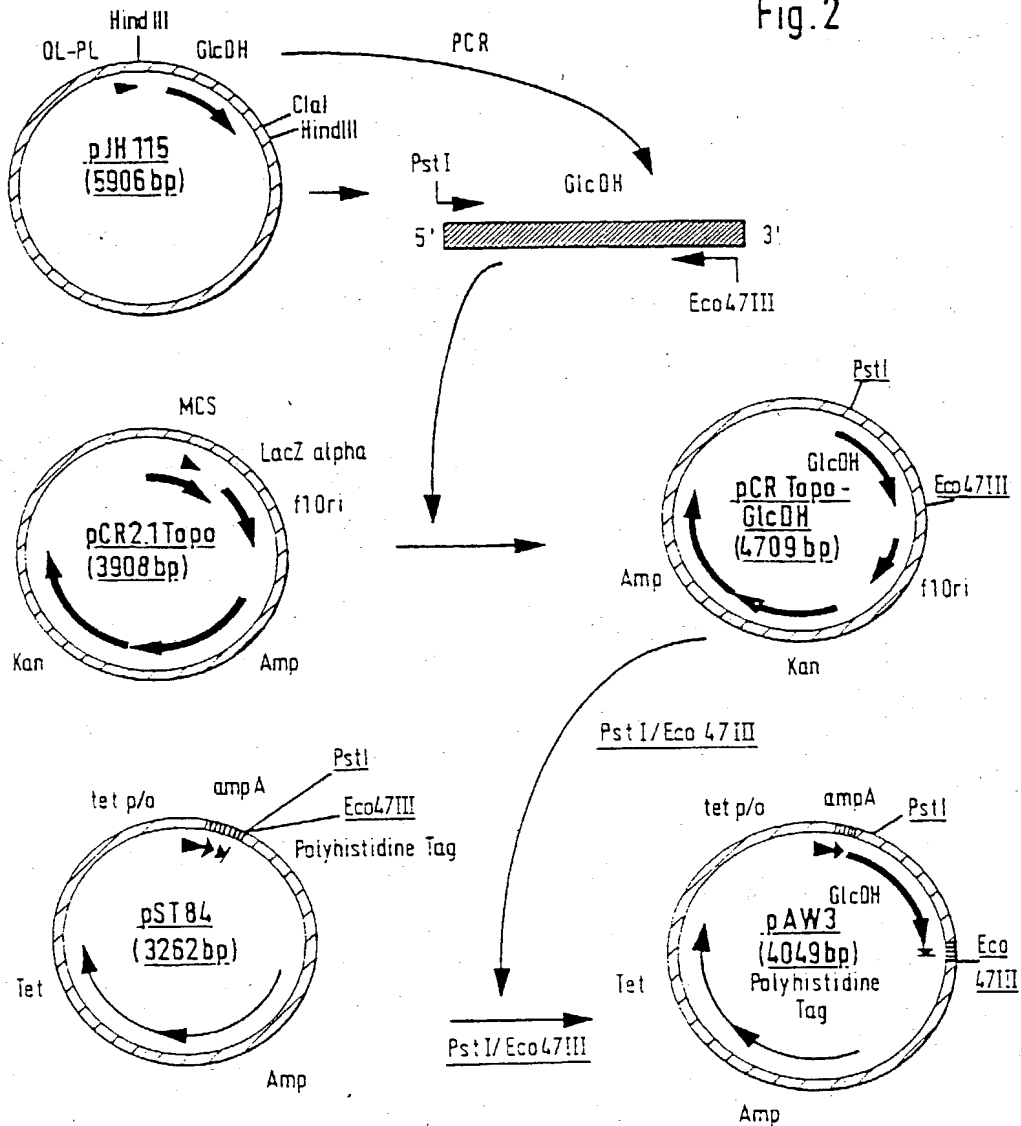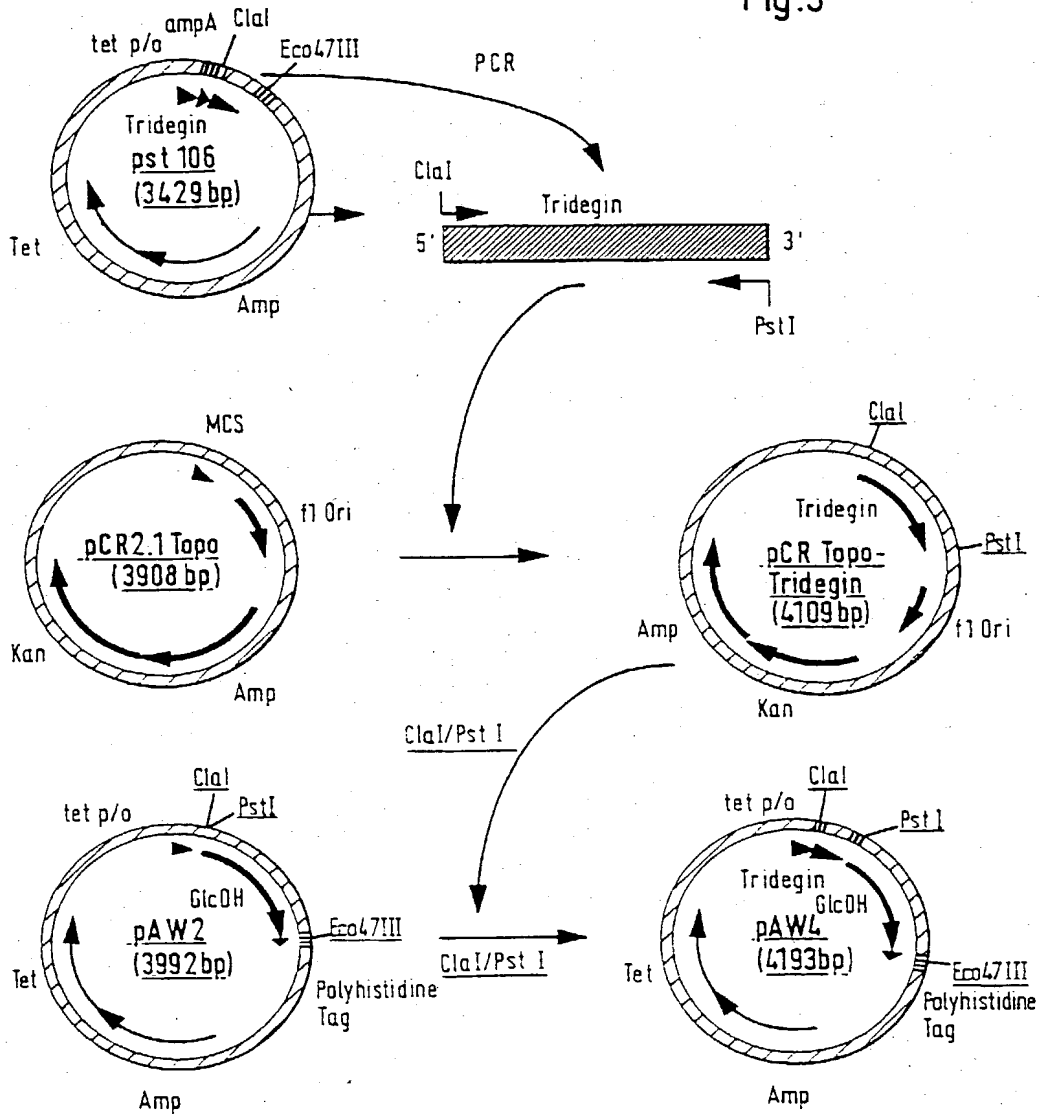
REPLACEMENT SHEET (RULE 26)

Fig. 2

Fig.3

Fig. 4

Fig. 5

kDa

49.5
34.8
28.3
20.4

GlcDH

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

the specification of which

(check one)

❑ is attached hereto.

❑x was filed on _____ as United States Application No. or PCT International

Application Number _____

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.
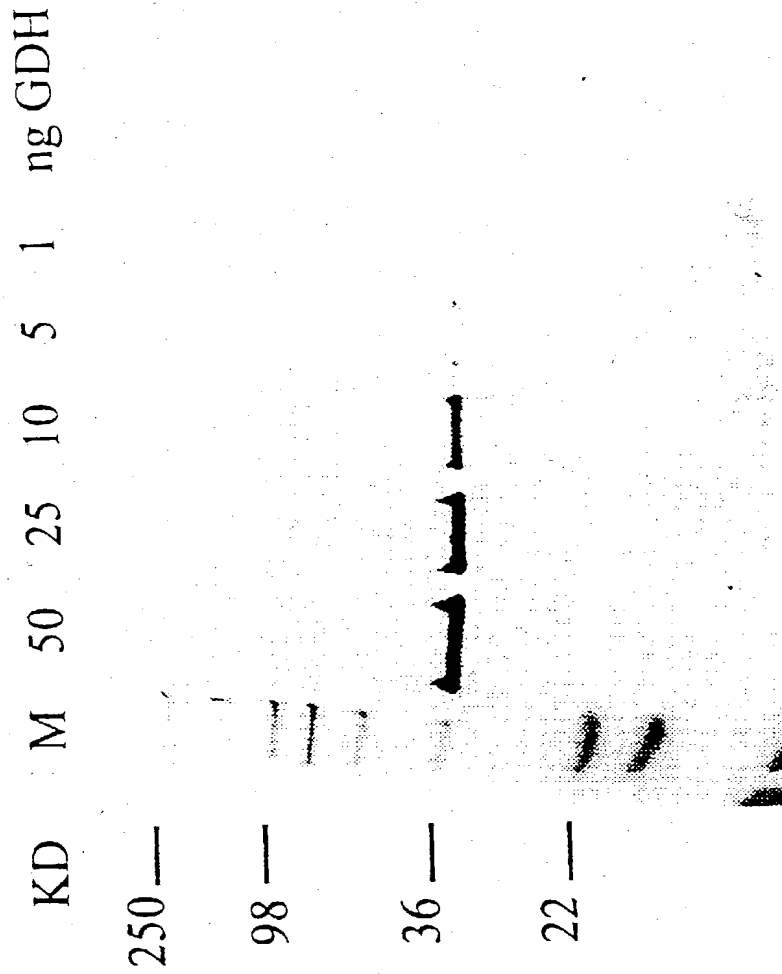
Prior Foreign Application(s)   Priority Not Claimed

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(Number)	(Country)	(Day/Month/Year Filed)	

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Ser Thr Gly Leu Gly Arg Ala Met Ala Val Arg Phe Gly Gln Glu Glu
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Ala Lys Lys Glu Val Glu Glu Ala Gly Gly Gln Ala Ile Ile Val Gln
          50   55   60

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His His His
270

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tgtagcggcg cattaagcgc ggcgggtgtg gtggttacgc gcagcgtgac cgctacactt  1170
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4

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gatcaccaag gtgcagagcc agccttctta ttcggccttg aattgatcat atgcggatta 3150

gaaaaacaac ttaaatgtga aagtgggtct taaaagcagc ataacctttt tccgtgatgg 3210

taacttcact agtttaaaag gatctaggtg aagatccttt ttgataatct catgaccaaa 3270

atcccttaac gtgagttttc gttccactga ccgtcagacc ccgtagaaaa gatcaaagga 3330

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ctaccagcgg tggtttgttt gccggatcaa gagctaccaa ctctttttcc gaaggtaact 3450

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cacttcaaga actctgtagc accgcctaca tacctcgctc tgctaatcct gttaccagtg 3570

gctgctgcca gtggcgataa gtcgtgtctt accgggttgg actcaagacg atagttaccg 3630

gataaggcgc agcggtcggg ctgaacgggg ggttcgtgca cacagcccag cttggagcga 3690

acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc cacgcttccc 3750

gaagggagaa aggcggacag gtatccggta agcggcaggg tcggaacagg agagcgcacg 3810

agggagcttc caggggggaa acgcctggta tctttatagtc ctgtcgggtt cgccaccttc 3870

tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg gaaaaacgcc 3930

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ca                                                              3992
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<210> 2
<211> 272
<212> PRT
<213> Bacillus megaterium (GlcDH-polytag fusion protein)

<400> 2

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 1               5                  10                  15

Thr Gly Leu Gly Arg Ala Met Ala Val Arg Phe Gly Gln Glu Glu Ala
                20                  25                  30

Lys Val Val Ile Asn Tyr Tyr Asn Asn Glu Glu Glu Ala Leu Asp Ala
            35                  40                  45

Lys Lys Glu Val Glu Glu Ala Gly Gly Gln Ala Ile Ile Val Gln Gly
        50                  55                  60

Asp Val Thr Lys Glu Glu Asp Val Val Asn Leu Val Gln Thr Ala Ile
    65                  70                  75                  80

Lys Glu Phe Gly Thr Leu Asp Val Met Ile Asn Asn Ala Gly Val Glu
                85                  90                  95
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5

Asn Pro Val Pro Ser His Glu Leu Ser Leu Asp Asn Trp Asn Lys Val
            100   105   110

Ile Asp Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg Glu Ala Ile
        115   120   125

Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly Asn Val Ile Asn Met Ser
    130   135   140

Ser Val His Glu Met Ile Pro Trp Pro Leu Phe Val His Tyr Ala Ala
145   150   155   160

Ser Lys Gly Gly Met Lys Leu Met Thr Glu Thr Leu Ala Leu Glu Tyr
                165   170   175

Ala Pro Lys Gly Ile Arg Val Asn Asn Ile Gly Pro Gly Ala Met Asn
            180   185   190

Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp Pro Glu Gln Arg Ala Asp
        195   200   205

Val Glu Ser Met Ile Pro Met Gly Tyr Ile Gly Lys Pro Glu Glu Val
    210   215   220

Ala Ala Val Ala Ala Phe Leu Ala Ser Ser Gln Ala Ser Tyr Val Thr
225   230   235   240

Gly Ile Thr Leu Phe Ala Asp Gly Gly Met Thr Lys Tyr Pro Ser Phe
                245   250   255

Gln Ala Gly Arg Gly Ala Met Arg Gly Ser His His His His His His
            260   265   270

<210> 3
<211> 4193
<212> DNA
<213> Bacillus megaterium + Heamenteria ghilianii fusion gene

<220>
<221> gene
<222> (1)..(4193)
<223> Plasmid PAW4

<220>
<221> CDS
<222> (141)..(344)
<223> Tridegin

<220>
<221> CDS
<222> (387)..(1169)
<223> Glucose Dehydrogenase

<220>
<221> CDS
<222> (1179)..(1211)
<223> Poly-histidine tag

<400> 3

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tattttacca ctccctatca gtgatagaga aaagtgaaat gaatagttcg acaaaaatct 120

agataacgag ggcaatcgat atg aaa cta ttg cct tgc aaa gaa tgg cat caa 173
                        Met Lys Leu Leu Pro Cys Lys Glu Trp His Gln
                          1   5   10

ggt att cct aac cct agg tgc tgg tgt ggg gct gat cta gaa tgc gca 221
Gly Ile Pro Asn Pro Arg Cys Trp Cys Gly Ala Asp Leu Glu Cys Ala
              15   20   25

caa gac caa tac tgt gcc ttc ata cct caa tgt aga cca aga tca gaa 269
Gln Asp Gln Tyr Cys Ala Phe Ile Pro Gln Cys Arg Pro Arg Ser Glu
          30   35   40

ctg att aaa cct atg gat gat ata tac caa aga cca gtc gag ttt cca 317
Leu Ile Lys Pro Met Asp Asp Ile Tyr Gln Arg Pro Val Glu Phe Pro
      45   50   55

aac cct cca tta aaa cct agg gag gaa agcgctatga gaggatcgca 364
Asn Leu Pro Leu Lys Pro Arg Glu Glu
 60   65

tcaccatcac catcacctgc ag atg tat aca gat tta aaa gat aaa gta gtt 416
                         Met Tyr Thr Asp Leu Lys Asp Lys Val Val
                          70   75

gta att aca ggt gga tca aca ggt tta gga cgc gca atg gct gtt cgt 464
Val Ile Thr Gly Gly Ser Thr Gly Leu Gly Arg Ala Met Ala Val Arg
      80   85   90

ttc ggt caa gaa gaa gca aaa gtt gtt att aac tat tac aac aat gaa 512
Phe Gly Gln Glu Glu Ala Lys Val Val Ile Asn Tyr Tyr Asn Asn Glu
 95   100   105   110

gaa gaa gct cta gat gcg aaa aaa gaa gta gaa gaa gca ggc gga caa 560
Glu Glu Ala Leu Asp Ala Lys Lys Glu Val Glu Glu Ala Gly Gly Gln
                  115   120   125

gca atc atc gtt caa ggc gat gta aca aaa gaa gaa gac gtt gta aat 608
Ala Ile Ile Val Gln Gly Asp Val Thr Lys Glu Glu Asp Val Val Asn
          130   135   140

ctt gtt caa aca gct att aaa gaa ttt ggt aca tta gac gta atg att 656
Leu Val Gln Thr Ala Ile Lys Glu Phe Gly Thr Leu Asp Val Met Ile
          145   150   155

aac aac gct ggt gtt gaa aac cca gtt cct tct cat gag cta tct cta 704
Asn Asn Ala Gly Val Glu Asn Pro Val Pro Ser His Glu Leu Ser Leu
          160   165   170

gat aac tgg aac aaa gtt att gat aca aac tta aca ggt gca ttc tta 752
Asp Asn Trp Asn Lys Val Ile Asp Thr Asn Leu Thr Gly Ala Phe Leu
175   180   185   190

gga agc cgt gaa gca att aaa tac ttc gtt gaa aac gac att aaa gga 800
Gly Ser Arg Glu Ala Ile Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly
              195   200   205

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aat gtt atc aac atg tct agc gtt cac gaa atg att cct tgg cca tta    848
Asn Val Ile Asn Met Ser Ser Val His Glu Met Ile Pro Trp Pro Leu
            210           215           220

ttt gtt cac tac gca gca agt aaa ggc ggt atg aaa cta atg acg gaa    896
Phe Val His Tyr Ala Ala Ser Lys Gly Gly Met Lys Leu Met Thr Glu
            225           230           235

aca ttg gct ctt gaa tat gcg cca aaa ggt att cgc gta aat aat att    944
Thr Leu Ala Leu Glu Tyr Ala Pro Lys Gly Ile Arg Val Asn Asn Ile
            240           245           250

gga cca ggt gcg atg aac aca cca att aac gca gag aaa ttt gca gat    992
Gly Pro Gly Ala Met Asn Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp
255           260           265           270

cca gaa caa cgt gca gac gta gaa agc atg att cca atg ggt tac atc   1040
Pro Glu Gln Arg Ala Asp Val Glu Ser Met Ile Pro Met Gly Tyr Ile
            275           280           285

ggt aaa cca gaa gaa gta gca gca gtt gca gca ttc tta gct tca tca   1088
Gly Lys Pro Glu Glu Val Ala Ala Val Ala Ala Phe Leu Ala Ser Ser
            290           295           300

caa gca agc tat gta aca ggt att aca tta ttt gca gat ggc ggt atg   1136
Gln Ala Ser Tyr Val Thr Gly Ile Thr Leu Phe Ala Asp Gly Gly Met
            305           310           315

acg aaa tac cct tct ttc caa gca gga aga ggc taatagagc gct atg aga 1187
Thr Lys Tyr Pro Ser Phe Gln Ala Gly Arg Gly              Ala Met Arg
            320           325                                  330

gga tcg cat cac cat cac cat cac taatagaagc ttgacctgtg aagtgaaaaa 1241
Gly Ser His His His His His His
            335           340

tggcgcacat tgtgcgacat tcttttgtc tgccgtttac cgctactgcg tcacggatct 1301

ccacgcgccc tgtagcggcg cattaagcgc ggcgggtgtg gtggttacgc gcagcgtgac 1361

cgctacactt gccagcgccc tagcgcccgc tcctttcgct ttcttccctt cctttctcgc 1421

cacgttcgcc ggctttcccc gtcaagctct aaatcggggg ctccctttag ggttccgatt 1481

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gccatcgccc tgatagacgg tttttcgccc tttgacgttg gagtccacgt tctttaatag 1601

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gagacaataa ccctgataaa tgcttcaata atattgaaaa aggaagagta tgagtattca 1901

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8

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9.

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cacgcttccc gaagggagaa aggcggacag gtatccggta agcggcaggg tcggaacagg 4001

agagcgcacg agggagcttc cagggggaaa cgcctggtat ctttatagtc ctgtcgggtt 4061

tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcagggggc ggagcctatg 4121

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<210> 4
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<212> PRT
<213> Bacillus megaterium + Heamenteria ghilianii fusion protein

<400> 4

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Arg Cys Trp Cys Gly Ala Asp Leu Glu Cys Ala Gln Asp Gln Tyr Cys
            20                  25                  30

Ala Phe Ile Pro Gln Cys Arg Pro Arg Ser Glu Leu Ile Lys Pro Met
        35                  40                  45

Asp Asp Ile Tyr Gln Arg Pro Val Glu Phe Pro Asn Leu Pro Leu Lys
    50                  55                  60

Pro Arg Glu Glu Met Tyr Thr Asp Leu Lys Asp Lys Val Val
55                  70                  75

Val Ile Thr Gly Gly Ser Thr Gly Leu Gly Arg Ala Met Ala Val Arg
    80                  85                  90

Phe Gly Gln Glu Glu Ala Lys Val Val Ile Asn Tyr Tyr Asn Asn Glu
95                  100                 105                 110

Glu Glu Ala Leu Asp Ala Lys Lys Glu Val Glu Glu Ala Gly Gly Gln
            115                 120                 125

Ala Ile Ile Val Gln Gly Asp Val Thr Lys Glu Glu Asp Val Val Asn
            130                 135             140

Leu Val Gln Thr Ala Ile Lys Glu Phe Gly Thr Leu Asp Val Met Ile
        145                 150                 155

Asn Asn Ala Gly Val Glu Asn Pro Val Pro Ser His Glu Leu Ser Leu
        160                 165                 170

Asp Asn Trp Asn Lys Val Ile Asp Thr Asn Leu Thr Gly Ala Phe Leu
175                 180                 185                 190

Gly Ser Arg Glu Ala Ile Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly
            195                 200                 205
```

Asn Val Ile Asn Met Ser Ser Val His Glu Met Ile Pro Trp Pro Leu
210   215   220

Phe Val His Tyr Ala Ala Ser Lys Gly Gly Met Lys Leu Met Thr Glu
        225   230   235

Thr Leu Ala Leu Glu Tyr Ala Pro Lys Gly Ile Arg Val Asn Asn Ile
    240   245   250

Gly Pro Gly Ala Met Asn Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp
255   260   265   270

Pro Glu Gln Arg Ala Asp Val Glu Ser Met Ile Pro Met Gly Tyr Ile
            275   280   285

Gly Lys Pro Glu Glu Val Ala Ala Val Ala Ala Phe Leu Ala Ser Ser
        290   295   300

Gln Ala Ser Tyr Val Thr Gly Ile Thr Leu Phe Ala Asp Gly Gly Met
        305   310   315

Thr Lys Tyr Pro Ser Phe Gln Ala Gly Arg Gly Ala Met Arg Gly Ser
    320   325   330
His His His His His His
335   340

<210> 5
<211> 32
<212> DNA
<213> Artificial sequence

<220>
<221> primer_bind
<222> (1)..(32)
<223> Primer 1, GlcDH

<220>
<223> Description of the artificial sequence: primer

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<210> 6
<211> 31
<212> DNA
<213> Artificial sequence

<220>
<221> primer_bind
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<223> Primer 2, GlcDH

<220>
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11

<210> 7
<211> 31
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: primer

<400> 7
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<210> 8
<211> 31
<212> DNA
<213> Artificial sequence

<220>
<221> primer_bind
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<223> Primer 4, GlcDH

<220>
<223> Description of the artificial sequence: primer

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<210> 9
<211> 31
<212> DNA
<213> Artificial sequence

<220>
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<223> Primer 5, Tridegin

<220>
<223> Description of the artificial sequence: primer

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<210> 10
<211> 31
<212> DNA
<213> Artificial sequence

<220>
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<223> Primer 6, Tridegin

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<223> Primer 7, pASK 75UPN

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<223> Description of the artificial sequence: primer

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<210> 12
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<220>
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<223> pASK 75 RPN

<220>
<223> Description of the artificial sequence: primer

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<211> 20
<212> DNA
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<222> (1)..(20)
<223> Primer 9, T7 Seq.

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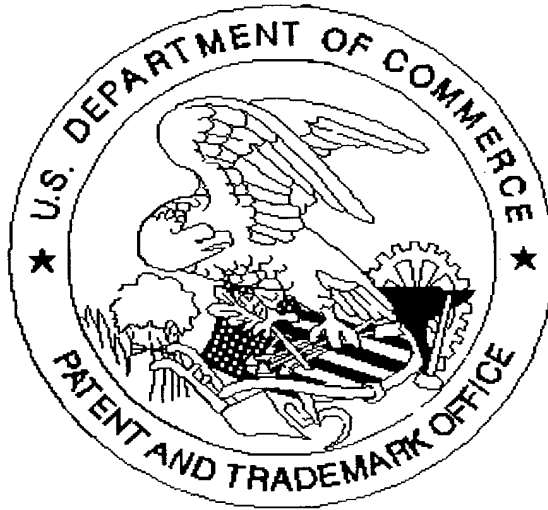
<400> 13
taatacgact cactataggg   20

<210> 14
<211> 18
<212> DNA
<213> Artificial sequence

<220>

13

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<221> primer_bind
<222> (1)..(18)
<223> Rev.  Seq.

<220>
<223> Description of the artificial sequence: primer

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tagaaggcac agtcgagg                                              18
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